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Cranial neural crest cells regulate head muscle patterning and differentiation during vertebrate embryogenesis

Ariel Rinon¹, Shlomi Lazar¹, Heather Marshall², Stine Büchmann-Møller³, Adi Neufeld¹, Hadas Elhanany-Tamir¹, Makoto M. Taketo⁴, Lukas Sommer³, Robb Krumlauf² and Eldad Tzahor^{1,*}

In the vertebrate head, mesoderm cells fuse together to form a myofiber, which is attached to specific cranial neural crest (CNC)-derived skeletal elements in a highly coordinated manner. Although it has long been recognized that CNC plays a role in the formation of the head musculature, the precise molecular underpinnings of this process remain elusive. In the present study we explored the nature of the crosstalk between CNC and mesoderm cells during head muscle development, employing three models for genetic perturbations of CNC development in mice, as well as experimental ablation of CNC in chick embryos. We demonstrate that although early myogenesis is CNC-independent, the migration, patterning and differentiation of muscle precursors are regulated by CNC. In the absence of CNC cells, accumulated myoblasts are kept in a proliferative state, presumably because of an increase of *Fgf8* in adjacent tissues, which leads to abnormalities in both differentiation and subsequent myofiber organization in the head. These results have uncovered a surprising degree of complexity and multiple distinct roles for CNC in the patterning and differentiation of muscles during craniofacial development. We suggest that CNC cells control craniofacial development by regulating positional interactions with mesoderm-derived muscle progenitors that together shape the cranial musculoskeletal architecture in vertebrate embryos.

KEY WORDS: Skeletal myogenesis, Cranial neural crest, Cranial paraxial mesoderm, Mouse, Chick

INTRODUCTION

Craniofacial development requires orchestrated communication between multiple specialized cell types. The ‘lead actors’ in this process are progenitor cells derived from the cranial paraxial mesoderm (CPM), as well as cells derived from the neural ectoderm, the cranial neural crest (CNC) (Helms et al., 2005; Trainor and Krumlauf, 2001). CNC differentiates into a wide variety of cell types, including neurons, glia, and pigment cells. These cells differ from trunk neural crest cells in various respects; most importantly, in their ability to give rise to the skeletal elements of the head (Le Douarin and Kalcheim, 1999). CPM located anterior to the somites provides the precursors for cranial skeletal muscles.

Both CPM and CNC cells stream into the neighboring branchial arches (BAs, also known as pharyngeal arches), which form the templates of adult craniofacial structures (Noden and Trainor, 2005). Within the BAs, CNC cells surround the muscle anlagen in a highly organized fashion, thereby separating the myoblasts from the overlying surface ectoderm (Hacker and Guthrie, 1998; Noden, 1983b; Trainor and Tam, 1995; Trainor et al., 1994). Mesoderm-derived myoblast cells subsequently fuse together to form a myofiber, which is attached to a specific CNC-derived skeletal element, through CNC-derived connective tissue, in a precisely coordinated manner. However, the molecular mechanisms underlying head muscle patterning – myoblast guidance, positioning and connection to specific attachment sites – remain poorly understood. Furthermore, the degree to which skeletal muscle

specification, differentiation and patterning is intrinsic to muscle (mesoderm) progenitors or controlled by extrinsic environmental signals (e.g. CNC cells) is a fundamental embryological question.

Craniofacial shapes are amazingly diverse in vertebrates but also within species [e.g. dogs, birds (Helms et al., 2005)]. This diversity apparently reflects a tight linkage between the skeletal elements (CNC), connective tissue (CNC) and skeletal muscle (mesoderm). Indeed, it has long been suggested that in addition to contributing to the formation of skeletal elements and connective tissue in the head, CNC cells may also be involved in the patterning of the head musculature (Couly et al., 1992; Ericsson et al., 2004; Grammatopoulos et al., 2000; Kontges and Lumsden, 1996; Noden, 1983a; Noden, 1983b; Olsson et al., 2001; Schilling and Kimmel, 1997).

Because skeletal muscles in the head still form (albeit in a distorted fashion) following *in vivo* ablation of the CNC cells in amphibian and chick embryos (Ericsson et al., 2004; Olsson et al., 2001; Tzahor et al., 2003; von Scheven et al., 2006) (reviewed in Noden and Trainor, 2005), the precise impact of CNC cells on head muscle formation remains unclear. Several genetic knockout models in mice have provided insights into CNC development, however; the link between these genetic perturbations and cranial muscle formation has not been explored. Thus, although it is generally accepted that CNC influences cranial muscle formation, exactly how CNC cells participate in this process remains to be elucidated.

Previously, we identified signals that regulate head muscle differentiation (Tzahor et al., 2003). In the head, both bone morphogenetic protein (BMP) and the canonical Wnt signaling molecules secreted by the dorsal neural tube act to repress skeletal muscle formation. This may occur via inhibition of the myogenic differentiation of the CPM in the vicinity of the neural tube. By contrast, these same Wnt ligands are required to stimulate myogenesis in the trunk. Moreover, CNC cells secrete both BMP inhibitors (Noggin, Gremlin) and Wnt inhibitors (Frzb), which together induce myogenic differentiation of the CPM *in vitro*

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(Tzahor et al., 2003). Therefore, head muscle differentiation is subject to a complex balance between neural tube-derived inhibitors and CNC-derived activators.

In the present study, we aimed to deepen our understanding of skeletal muscle development in the vertebrate head by focusing on the molecular crosstalk between CNC and mesoderm progenitors during vertebrate craniofacial development. To do so, we used genetic perturbations of CNC development in mouse embryos (Chen and Behringer, 1995; Gavalas et al., 2001; Lee et al., 2004) and cellular perturbations of CNC development in avian embryos. Our results indicate that although CNC cells are not necessary for the early specification of skeletal muscle progenitors, they play a crucial role in the migration, positioning and differentiation of cranial muscle precursors in vertebrate embryos. Our findings also demonstrate that in the absence of CNC cells, other tissues and signals are capable of promoting skeletal muscle differentiation in the head.

MATERIALS AND METHODS

Mouse embryos

References for the mouse lines that were used are specified in the text.

Chick and quail embryos

Fertilized chicken and quail eggs were incubated for 1–5 days at 38.5°C in a humidified incubator up to Hamburger–Hamilton stages 8–26.

CNC ablation

Dorsal neural tube ablation was performed at around stage 8, as previously described (Tzahor et al., 2003).

In situ hybridization and histological analyses

A full list of the in situ hybridization probes and detailed protocols are available upon request (see also Tirosh-Finkel et al., 2006).

Cell proliferation assay combined with in situ hybridization

Stage 8 chick embryos were incubated for ~45 hours, followed by the addition of 200 µl of 10 mM 5'-bromo-2'-deoxyuridine (BrdU) for 1 hour in ovo. Thereafter, embryos were fixed and processed for in situ hybridization and sectioning. Selected sections were subjected to an immunostaining protocol (Tirosh-Finkel et al., 2006). BrdU-positive cells in the myogenic core, demarcated by *Myf5* staining, were counted and divided by the total number of DAPI-positive nuclei in the same region.

In-ovo dye injection

DiI, CM-DiI or DiO labeling experiments were performed on stage 8 chick embryos (Tirosh-Finkel et al., 2006).

RESULTS

Cranial myogenesis is initiated in the absence of CNC cells

To label muscle cells at discrete stages of myogenesis we employed the following hierarchy of markers: capsulin [also known as *Tcf21* – Mouse Genome Informatics (MGI)], *Tbx1*, *Myf5*, *MyoD* (also known as *Myod1* – MGI), myogenin (*Mgn*), desmin and myosin heavy chain (*MHC*) (Tajbakhsh, 2005); *Dlx5* (in the mouse) was used to label the CNC. We began by investigating whether CNC cells are a prerequisite to the specification of the skeletal muscle lineage. Studies in amphibians (Ericsson et al., 2004; Olsson et al., 2001) as well as our previous work with chick embryos (Tzahor et al., 2003) suggest that early steps of muscle development in the vertebrate head are CNC-independent. However, this interpretation is complicated by the fact that neural crest cells in chick embryos are known to regenerate following extirpation (Saldivar et al., 1997; Scherson et al., 1993; Vaglia and Hall, 1999).

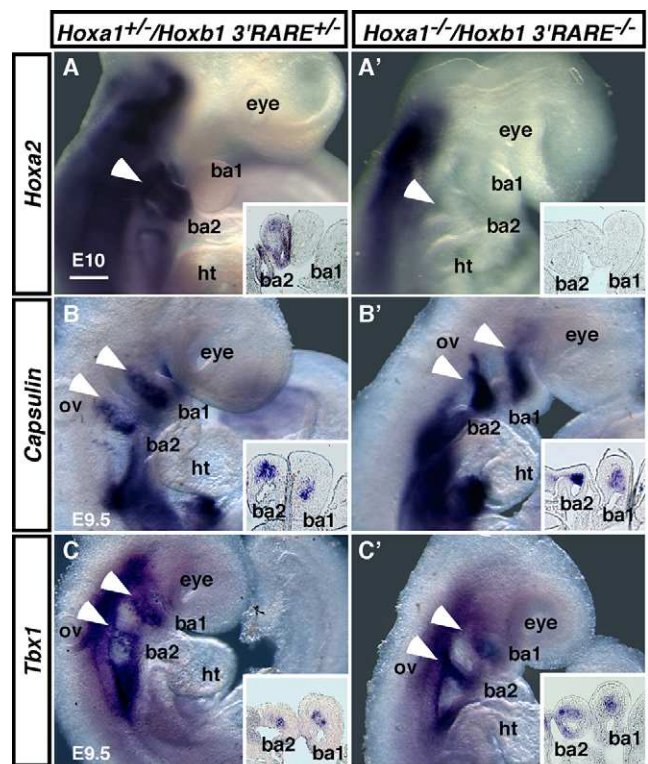


Fig. 1. CNC cells are not necessary for early myogenesis in *Hoxa1/Hoxb1-3'RARE* double-mutant embryos. (A,A') Whole-mount in situ hybridization (ISH) for *Hoxa2* and the corresponding coronal section (inset) of control and double mutant, $n=1$. (B,B') Capsulin ISH in control and mutant embryos, $n=3$. (C,C') *Tbx1* ISH in control and the mutant embryos, $n=3$. Arrowheads point to the branchial arches (ba1 and ba2); ht, heart. Scale bars: in A, 0.36 mm for A'-C'.

Therefore, we used a mouse model involving a genetic loss in a specific subset of CNC cells. In the mouse, previous studies of combined *Hoxa1/Hoxb1* mutants revealed extensive synergy between these two genes. The combination of a homozygous null *Hoxa1* allele (Lufkin et al., 1991) and a homozygous *Hoxb1-3'RARE* allele, a mutant of the retinoic acid enhancer required for *Hoxb1* expression in the neural tube (Marshall et al., 1994), resulted in the specific failure of CNC cells to form and migrate into the second branchial arch (BA2), whereas CNC cells in the other BAs remained unaffected (Gavalas et al., 2001). This genetic ablation of CNC cells in BA2 did not significantly affect the early patterning of endoderm and surface ectoderm in the arch; however, its effect on mesodermal cells was not examined.

The *Hoxa1/Hoxb1-3'RARE* double-knockout mouse model therefore provides means to determine whether CNC cells are required for early myogenesis. In order to confirm the mutant BA2 phenotype, we performed in situ hybridization for *Hoxa2* (Fig. 1A; note the specific loss of *Hoxa2* expression in BA2 of the double-mutant embryo in A'). Next, control (double heterozygote) or mutant (double homozygote) E9.5 mouse embryos were subjected to in situ hybridization for the early skeletal muscle markers capsulin and *Tbx1* [Fig. 1B,C (Kelly et al., 2004; Lu et al., 2002)]. Because these early skeletal muscle markers were detected in BA2 of mutant embryos, we propose that CNC cells are not necessary for the early stages of head muscle specification.

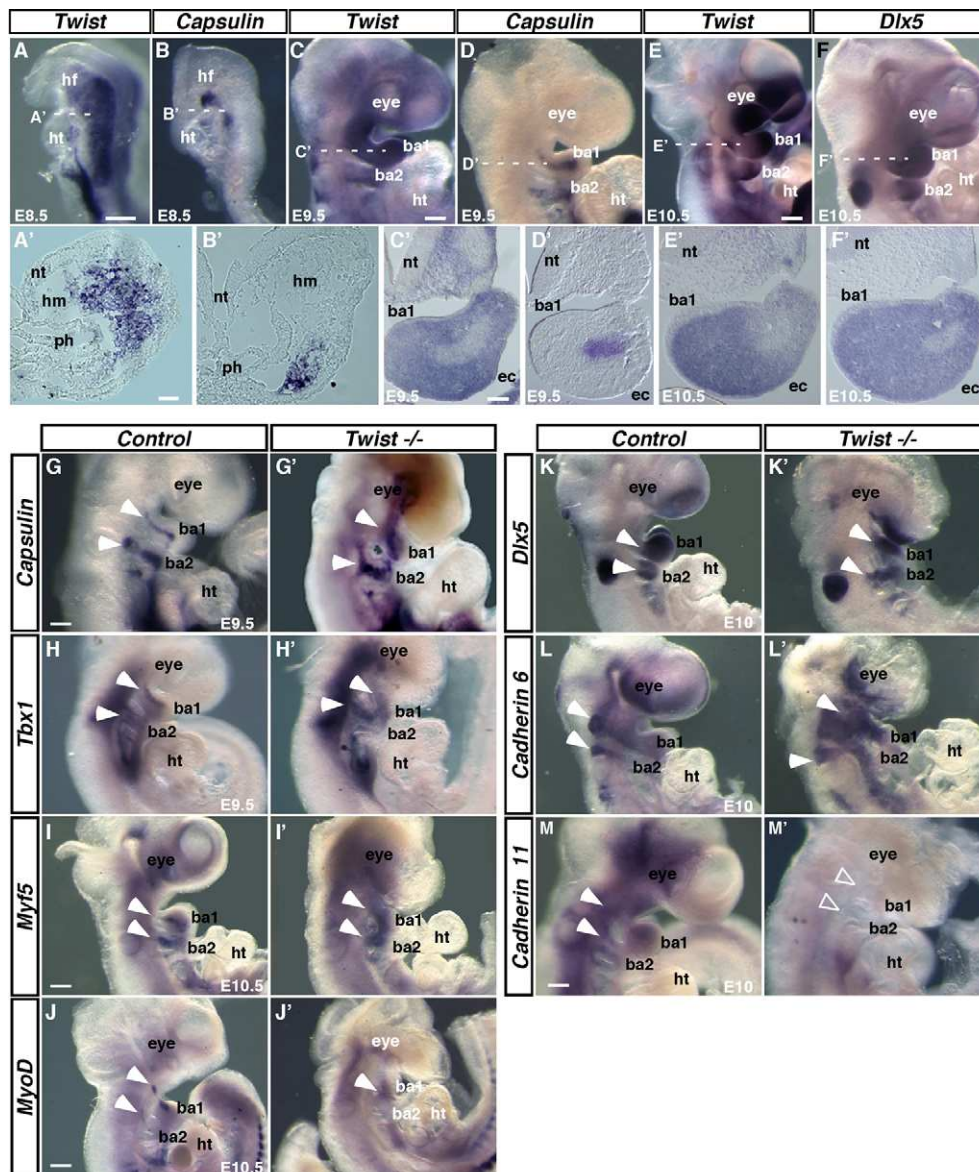


Fig. 2. Defects in cranial muscle patterning in *Twist* mutant embryos. (A-F') A comparative expression analysis for cranial mesoderm and neural crest markers in mouse embryos. Whole-mount and section in situ hybridization for *Twist* (A,A',C,C',E,E'), capsulin (B,B',D,D') and *Dlx5* (F,F'). Broken lines indicate plane of section. (G,G') Whole-mount in situ hybridization (ISH) for capsulin in control and *Twist* mutants, $n=6$. Arrowheads point to the muscle anlagen in the branchial arches (ba1 and ba2). (H,H') *Tbx1* ISH, $n=7$. (I,I') *Myf5* ISH, $n=7$. (J,J') *MyoD* ISH, $n=7$. (K-M') *Dlx5*, cadherin 6 and cadherin 11 expression patterns in control and *Twist* mutants, $n=5$. Arrowheads point to the CNC cells in the branchial arches (ba1 and ba2) and open arrowheads indicate their absence. hf, head fold; hm, head mesenchyme; ht, heart; nt, neural tube; ph, pharynx. Scale bars: in A, 0.41 mm for B; in A', 0.25 mm for B'; in C and G, 0.3 mm for D,G',H-H',I',J',K',L',M'; in C' and E, 0.45 mm for D',E',F-F',I,I',K,K,L,M.

Muscle patterning defects could be observed in the *Hoxa1/Hoxb1-3* *RARE* mutants: first, the expression patterns of capsulin and *Tbx1* in BA2 were slightly expanded in the mutant embryos, as compared with controls (Fig. 1B',C'), and second, capsulin expression in the myogenic core seemed to be more condensed, presumably because of the absence of infiltrating CNC cells within the core of BA2 (inset in Fig. 1B'). These results are consistent with a possible role for CNC during skeletal muscle patterning at later stages of muscle development.

Cranial muscle patterning and differentiation are perturbed in *Twist* and *CA- β -catenin/Wnt1-Cre* mutant embryos

Twist1 (referred to here as *Twist*) is a basic helix-loop-helix transcription factor involved in a diverse array of cell differentiation and morphogenic processes during gestation, as well as in post-embryonic development (O'Rourke and Tam, 2002). Of particular interest to us is the fact that this molecule is intricately involved in craniofacial development. The loss-of-function of *Twist* in mouse embryos has revealed its essential role in CNC migration and

differentiation, although mutant embryos do not survive beyond E10.5 (Chen and Behringer, 1995; Soo et al., 2002). Importantly, in *Twist* mutant embryos, CNC cells are formed at all levels of the brain (Fig. 2K'), despite the failure of the neural tube to close, but their patterns of migration had changed. In addition, it has been suggested that CNC development was arrested at an early phase of skeletogenic differentiation in *Twist* mutants (Soo et al., 2002).

In the mouse, at E8.5, *Twist* expression is detected in the head mesenchyme (Fig. 2A,A'), although its expression differed from that of the early myogenic marker capsulin (Fig. 2B,B',D,D'). From E9 onwards, *Twist* is expressed exclusively in CNC cells as shown by its overlapped expression pattern with the CNC marker *Dlx5* (Fig. 2E,E',F,F', and data not shown). Therefore, we used the *Twist* mouse model to determine its indirect impact on skeletal muscle formation. In situ hybridization of control and *Twist* mutants at E9.5-10.5 indicated a profound *Twist*-dependent alteration in the expression of the skeletal muscle markers capsulin, *Tbx1*, *Myf5* and *MyoD* (Fig. 2G-J'). In control embryos, muscle markers are typically expressed in the core of the BAs (Fig. 2G,H,I,J); this pattern of expression was altered in *Twist* mutants (Fig. 2G',H',I',J'). These analyses indicate

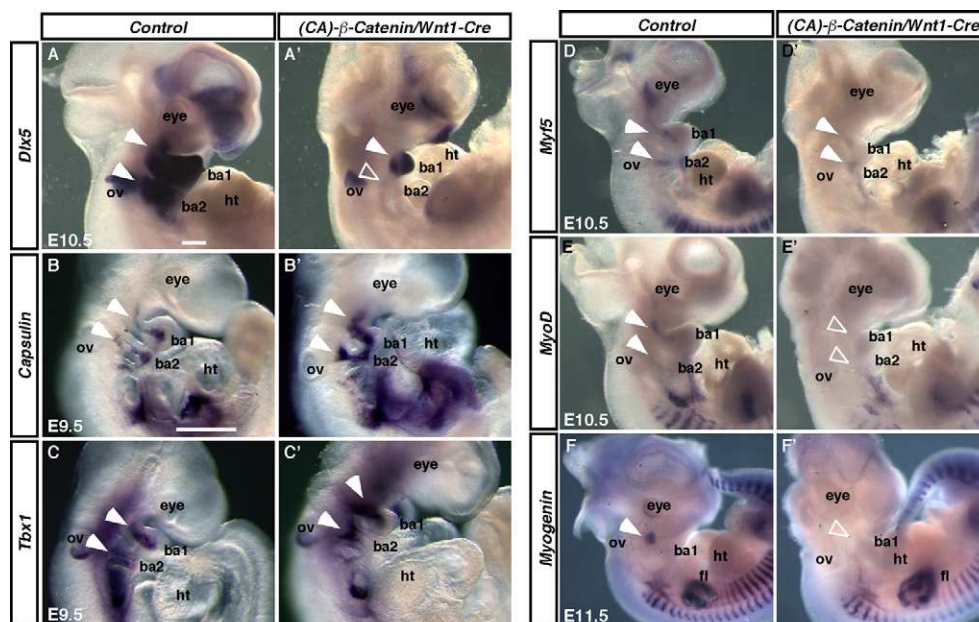


Fig. 3. Muscle patterning and differentiation defects in CA- β -catenin/*Wnt1*-Cre mutant embryos. (A,A') Whole-mount in situ hybridization (ISH) for *Dlx5* in control and CA- β -catenin/*Wnt1*-Cre mutants, $n=3$. Arrowheads point to the CNC cells in the branchial arches (ba1 and ba2). (B,B') Capsulin ISH in control and mutants, $n=6$. Arrowheads point to the muscle anlagen in the branchial arches. (C,C') *Tbx1* ISH, $n=6$. (D,D') *Myf5* ISH, $n=5$. (E,E') *MyoD* ISH, $n=4$. (F,F') Myogenin ISH, $n=4$. Open arrowheads indicate on the loss of muscle anlagen. fl, forelimb; ht, heart; nt, neural tube. Scale bars: in A, 0.5 mm for A', D-D', E-E', F-F'; in B, 0.7 mm for B'-C'.

that patterning and differentiation of CNC cells are tightly linked to those of the skeletal muscle precursors, but they are dispensable for initial myogenic specification.

Because it was recently shown that Twist directly regulates the expression of members of the cadherin family of adhesion molecules during tumor development and metastasis (Yang et al., 2004), we analyzed the distribution of the cadherin mRNAs in the CPM and CNC (Fig. 2L,M). The expression patterns of both cadherin 6 (Fig. 2L') and cadherin 11 (Fig. 2M') (see also Soo et al., 2002) were severely altered in *Twist* mutants compared with the controls. Taken together, these findings suggest that head muscle patterning is regulated non-cell-autonomously by Twist, expressed by CNC cells. This regulation may also involve cadherin molecules expressed by both CNC and mesoderm cells.

Wnt signaling has previously been implicated in the early stages of neural crest development [e.g. neural crest induction and lineage specification (Garcia-Castro et al., 2002; Ikeya et al., 1997)]. Activation of the Wnt signaling pathway in mice by expressing a stabilized β -catenin in neural crest cells (using the *Wnt1-Cre* transgene) promoted the development of sensory neurons, at the expense of other neural crest derivatives (Lee et al., 2004). *Dlx5* is thought to be expressed in CNC-derived skeletogenic progenitors (Holleville et al., 2003); its expression was markedly reduced in BA1 and was undetectable in BA2 of E10.5 mutant [constitutively active (CA)- β -catenin/*Wnt1-Cre*] embryos compared with controls (Fig. 3A,A'), supporting the findings of Lee et al. (Lee et al., 2004). Accordingly, we used this mouse model to determine how the forced differentiation of CNC cells into the neuronal lineage affects skeletal muscle formation in mouse embryos. In contrast to the typical expression of capsulin in the core of the BAs in control embryos at E9.5 (Fig. 3B), capsulin expression was severely mispatterned and upregulated in the mutant (Fig. 3B'). Likewise, the expression of *Tbx1* was upregulated in the core of the mutants' BA1, as compared with that of control embryos (Fig. 3C,C').

We next explored myogenic determination and differentiation at later developmental stages in both control and CA- β -catenin/*Wnt1-Cre* embryos. At E10.5, we detected traces of *Myf5* expression in the mutants' BAs compared with the controls (Fig. 3D,D'); *MyoD* expression was undetectable in those mutants (Fig. 3E,E').

Consistent with the loss of *MyoD* in the head musculature, expression of *Mgn* in cranial muscles was also undetectable at E11.5 (Fig. 3F,F'). These findings indicate that abnormal CNC fate determination can lead to defects in patterning and differentiation of muscle precursors in the head. Taken together, our analyses in mouse models suggest that CNC cells are not necessary for the initial specification of the head muscle progenitors; however, they play key roles in regulating the patterning and differentiation of the cranial skeletal muscles during later stages of myogenesis.

Ablation of the CNC cells in chick embryos alters myogenic gene expression

To complement our mouse genetic studies, we extended this analysis to avian embryos. Our previous finding in chick embryos that *Myf5* was expressed following CNC ablation (Tzahor et al., 2003) led us to consider that CNC ablation in the chick may impact the patterning and/or the kinetics of myogenesis (presumably downstream of *Myf5*). We employed the CNC-ablation model in stage 8 chick embryos (Tzahor et al., 2003) (Fig. 4A,A'). After 36–48 hours, embryos were subjected to in situ hybridization for the muscle markers capsulin, *Tbx1*, *Myf5* and *MyoD* (Fig. 4). Expression of *Tbx1* and *Myf5* in the BAs was upregulated and expanded to fill the entire arch mesenchyme (Fig. 4B,D). Capsulin expression was detected between the BAs in the operated embryos (Fig. 4C'). *MyoD* was slightly upregulated in the proximal region of BA1 (maxilla) in these embryos, whereas its expression in the distal arch (mandible) and in BA2 was diminished (Fig. 4E'). Our findings indicate that the expression of the skeletal muscle markers was maintained following CNC ablation in chick embryos. However, removal of the CNC in chick embryos severely distorted the expression patterns of myogenic genes. These results corroborate our findings in the mouse mutant embryos, and suggest that the nature of these interactions is conserved in vertebrates.

CNC cells influence mesoderm migration and axial registration

Because both CNC and CPM migrate en route to the BAs via overlapping migratory pathways (Hacker and Guthrie, 1998; Trainor and Tam, 1995), we explored the idea that CNC cells might influence

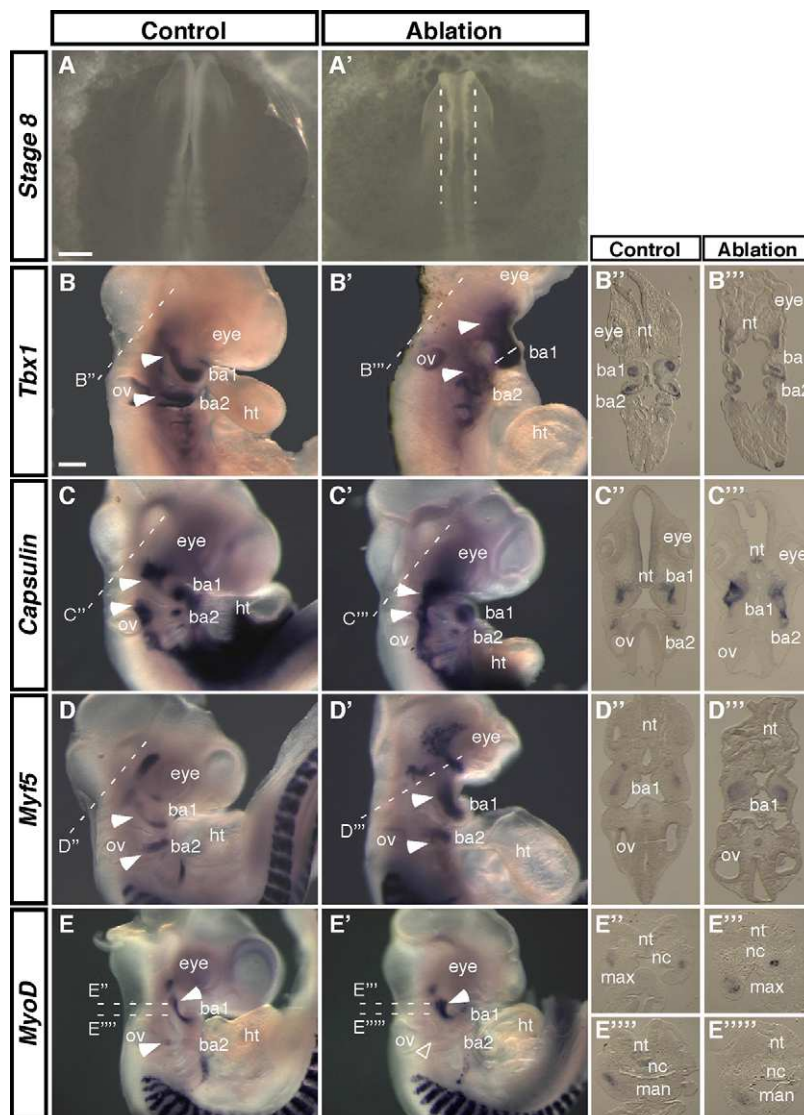


Fig. 4. Ablation of the CNC in chick embryos affects cranial muscle patterning. Images of control (A) or CNC-ablated (A') chick embryos at stage 8 (ablation boundaries are marked by white broken lines in A', dorsal view). (B-E') Whole-mount in situ hybridization in control and CNC-ablated embryos (lateral views) and the coronal/transverse sections (white broken line) of the corresponding embryos on the left: *Tbx1* (B-B''', $n=7/7$), capsulin (C-C''', $n=5/6$), *Myf5* (D-D''', $n=8/9$) and *MyoD* (E-E''', $n=5/5$). Arrowheads point to the muscle anlagen in the branchial arches (ba1 and ba2) and open arrowhead indicates their absence. ht, heart; nt, neural tube; ov, otic vesicle. Scale bars: in A, 0.4 mm for A'; in B, 0.5 mm for B'-E'.

the migration of mesodermal cells. In order to gain insights into these migratory events, DiI was used as a lineage tracer to unilaterally label the CPM at stage 8 in both control and CNC-ablated embryos (Fig. 5A,B). In control embryos, DiI-labeled mesodermal cells migrated in a typical crescent-shaped pattern into BA1, whereas in CNC-ablated embryos some mesoderm cells failed to enter BA1 (Fig. 5B'). Cell death was not observed in the CNC-ablated embryos, indicating that these cells were not lost because of increased apoptosis (data not shown). In view of the robust expression of mesodermal markers in BA1 of CNC-ablated embryos (see *Tbx1* and *Myf5* expression data, Fig. 4), and the reduced migration of mesodermal cells into BA1 (Fig. 5), we speculated that in the absence of CNC cells, other mesodermal cells are able to enter BA1.

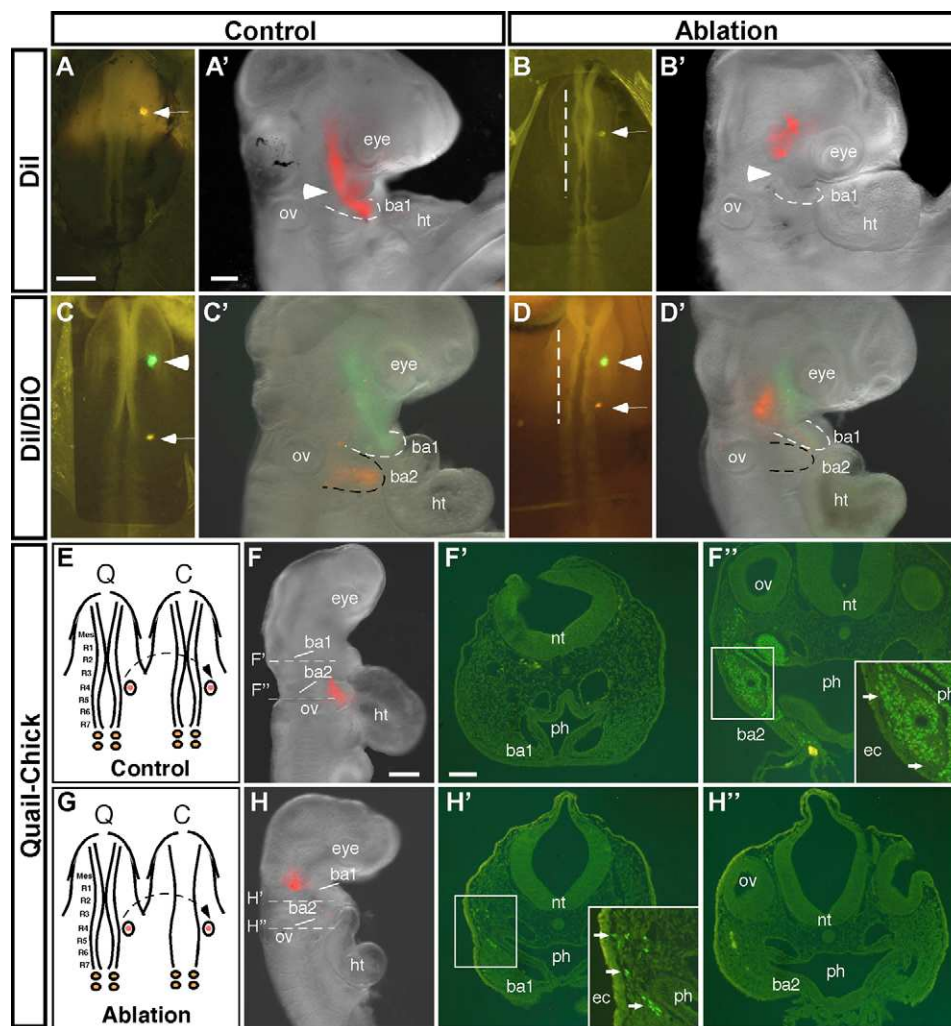
To test this possibility, we labeled the prospective BA1 and BA2 CPM in stage 8 chick embryos with DiO and DiI, respectively (Fig. 5C,D). In control embryos injected with both dyes, separate streams of CPM were detected where DiO-labeled cells enter BA1, and DiI-labeled cells enter BA2. In the CNC-ablated embryos, however, these two migratory streams fuse, and partially enter BA1 (Fig. 5C',D'). Thus, both the migratory pathways and the anterior-posterior registration of the CPM seem to be affected by the absence of the CNC.

To confirm these findings by another approach, we employed quail-chick transplantations in conjunction with fate mapping labeling (Fig. 5E,F,G,H). Quail CPM cells at the level of rhombomere 4, which normally migrate to BA2 (Hacker and Guthrie, 1998; Trainor and Tam, 1995), were labeled with DiI, transplanted into a stage-matched chick embryo, and left to develop for an additional 24 hours. Cells from the control graft migrated into BA2, as shown in both whole-mount embryos and sections stained with quail-specific QCPN antibodies (Fig. 5F). In the absence of the CNC, CPM cells failed to enter BA2 (Fig. 5H). Instead, some grafted cells shifted anteriorly toward BA1 (Fig. 5H'). In addition, QCPN-labeled cells in the ablated embryo were located more dorsally, compared with quail-derived mesoderm cells in control embryos (Fig. 5, compare panels F'' with H'). Taken together, these different approaches demonstrate that CNC cells regulate the migration and axial registration of CPM cells en route to the BAs.

An additional mechanism that could account for the upregulation of *Myf5* in the BAs of CNC-ablated embryos (Fig. 4) involves increased myoblast proliferation. To explore how ablation of the CNC affects mesoderm proliferation, we performed in situ hybridization for *Myf5* followed by BrdU immunostaining on transverse sections (Fig. 6A-D). In the trunk, *Myf5*-expressing cells

Fig. 5. CNC cells influence paraxial mesoderm migration and axial registration in chick embryos.

(A) An image of a stage 8 embryo injected with Dil in the CPM (arrow indicates dye location, dorsal view). (A') A lateral view of the embryo in A after 48 hours; arrowhead points to the labeled cells migrating toward BA1. (B,B') Dil labeling of the CPM in CNC-ablated embryos. In some ablated embryos cell migration was arrested ($n=8/13$) whereas in others partial migration towards BA1 was observed ($n=4/13$) compared with normal migration of CPM cells in controls ($n=13/14$). (C-D') Embryos were labeled with both Dil and DiO simultaneously (DiO, green, arrowheads in C and D; Dil, red, arrows in C and D). CPM cell migration was monitored after 48 hours. A mixture of the DiO- and Dil-labeled cells streaming toward BA1 is seen in the ablated embryo (D', $n=6/8$) compared with the separate streams seen in controls (C', $n=4/5$). Ablation boundaries are marked by broken line. (E-H') Quail-chick (Q-C) transplantation assay; E, a scheme of the experiment. Stage 8 quail CPM grafts labeled with Dil at the level of rhombomere 4 and then transplanted into stage-matched chick embryos. (F) A lateral view of the Q-C chimeric embryo after 24 hours. (F'-F'') Transverse sections through the BAs of the embryo on the left (F', ba1, F'', ba2) stained with the quail-specific antibody (QCPN, in green). Note the quail-derived cells exclusively in BA2 (F'', higher magnification in the inset, $n=4/4$). (G-H'') Similar images as shown in E-F' except that the host chick embryo was CNC ablated. In the CNC-ablated embryo, quail-derived cells are seen in BA1 (arrows in H', inset, $n=3/4$) but not in BA2. Lateral views of embryos (A',B',C',D',F,H) are shown as an overlay of bright field and fluorescence images. ec, ectoderm; ht, heart; nt, neural tube; ov, otic vesicle; ph, pharynx. Scale bars: in A, 0.4 mm for B,C,D; in A', 0.5 mm for B',C',D'; in F, 0.36 mm for H; in F', 0.2 mm for H' and 0.1 mm for F'',H''.



in the myotome were mostly BrdU-negative (Fig. 6A''), indicating that these cells underwent myogenic differentiation. In a similar manner, myogenic cells in the core of the BAs seemed to be BrdU-negative in control embryos (Fig. 6B'', quantified in D). In sharp contrast, BrdU staining in the myogenic cores of CNC-ablated BAs was significantly increased (Fig. 6C'',D). Similar results were obtained using immunofluorescence analysis for Myf5 and BrdU in the myotome (Fig. 6E-G'''), in BA2 of control (Fig. 6F-F'''), or in CNC-ablated embryos (Fig. 6G-G'''). These analyses revealed that there are more Myf5⁺/BrdU⁺ cells in BA2 of CNC-ablated embryos than in the control (note the yellow spots in Fig. 6G'''). We propose that in the absence of CNC cells, proliferating myoblasts (Myf5⁺/BrdU⁺) accumulate in the BAs. These cells apparently fail to exit the cell cycle and thus skeletal muscle differentiation may be reduced or delayed.

Recent studies demonstrated that cardiac neural crest ablation in chick embryos led to an *Fgf8*-dependent increase in mesoderm proliferation in the secondary heart field (Hutson et al., 2006; Waldo et al., 2005). Along these same lines, significant upregulation of *Fgf8* could be observed in the ventral (distal) ectoderm of BA1 (in

both maxilla and mandible) and in the BA2 of the CNC-ablated embryos (Fig. 6H,H',I,I'). Furthermore, application of *Fgf8* protein to CPM explants in vitro reduced myogenic differentiation (Fig. 6J). It appears that other signaling pathways were deregulated as a result of CNC ablation, as indicated by the moderate upregulation of both *Frzb* and *Bmp4* in the BA ectoderm (data not shown). We propose that these changes in signaling molecules in the BA ectoderm following CNC ablation in chick embryos can increase myogenic cell proliferation, resulting in delayed or reduced differentiation of the branchiomic musculature.

To gain a deeper understanding of the effect(s) of CNC cells on mesodermal cell proliferation/differentiation, we analyzed control and CNC-ablated chick embryos, using a combined in vitro-in vivo approach (Fig. 6K). Ablation of the CNC was performed at stage 8 and the embryos were left to develop in ovo until stage 10. Explants of the CPM (including the ectoderm and endoderm) were then dissected from these embryos and assayed by RT-PCR after 4 days. The reduced levels of the CNC markers *Noelin* and *Frzb* indicated that the ablation was successful. *MyoD*, *Mgn* and *MHC* were reduced in the CNC-ablated embryos, compared with their levels in the controls,

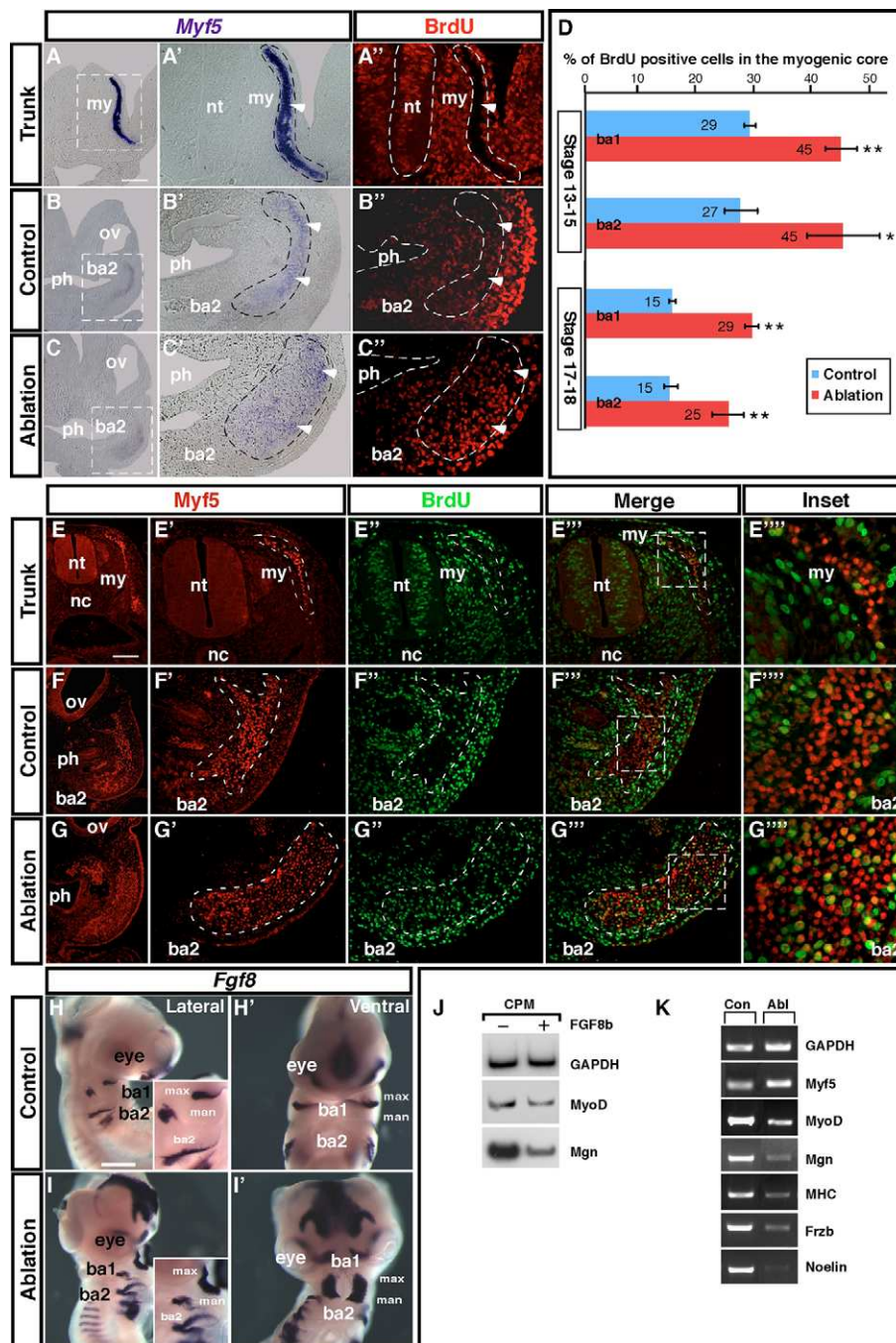


Fig. 6. Increased cell proliferation and *Fgf8* upregulation after CNC ablation in chick embryos. (A-C'') A combined in situ hybridization for *Myf5* (purple) and BrdU staining (red) in transverse sections of stage 18 embryos in the trunk (A-A'') and BA2 region (B-B'', control) or CNC-ablated embryo (C-C''). Arrowheads point to *Myf5* expression and BrdU staining in the muscle anlagen. (D) A quantification analysis of proliferating myoblasts in branchial arches of control and CNC-ablated embryos at 26 and 45 hours of incubation. Similar results were obtained in three independent experiments and in each experiment the bars represent counts from three adjacent sections. * $P < 0.05$, ** $P < 0.01$. (E-G'') A double immunostaining for *Myf5* (red) and BrdU (green). (E-E'') A transverse section at the trunk level. (F-F'') A transverse section at the level of BA2 in control embryos. (G-G'') A transverse section at the level of BA2 in CNC-ablated embryos. Merged BrdU/*Myf5* images plus higher magnifications (inset) are shown on the right. In situ hybridization for *Fgf8* in control (H,H'') or CNC-ablated embryos (I,I''). (J) RT-PCR results of CPM explants from control or 100 ng/ml FGF8b-treated explants incubated for 3 days in culture, $n = 2/2$. (K) RT-PCR results of CPM explants from control or CNC-ablated embryos. Whereas CPM explants underwent myogenesis in control cultures, a reduction in myogenesis was observed following CNC ablation ($n = 4/4$). my, myotome; nt, neural tube; ov, otic vesicle; ph, pharynx. Scale bars: in A and E, 0.15 mm for B,C,F,G and 75 μ m for A'-A'',B'-B'',C'-C'',E'-E'',F'-F'',G'-G''; in H, 0.5 mm for H'-I'.

whereas *Myf5* was slightly upregulated in the ablated embryos (Fig. 6K), in line with the upregulation of *Myf5* after CNC ablation in vivo (Fig. 4). These results further suggest that CNC cells exert their effect on myogenic differentiation downstream of *Myf5*.

The expression of myogenic regulatory factors (e.g. *Myf5*) represents cell specification to the myogenic lineage, whereas expression of desmin and, at later embryonic stages, MHC reflects the subsequent determination and differentiation of skeletal muscle progenitors to muscle fibers [Fig. 7A, adapted from Tajbakhsh (Tajbakhsh, 2005)]. To study the impact of CNC cells on head muscle differentiation in vivo, we followed this timetable of differentiation by performing immunofluorescence staining for desmin, MHC and F-actin on sections from both control and CNC-

ablated chick embryos at E4.5-5. In control embryos, desmin and MHC were detected in BA1-derived jaw muscle (e.g. intermandibular and mandibular adductor muscles, Fig. 7B,D,F). Higher magnifications of these sections revealed a scaffold of skeletal muscle progenitors, with their subsequent organization into myofibers in the control embryos (Fig. 7B'',D'',F'',H'',J''). However, in the CNC-ablated embryos, we observed a dramatic reduction in myogenic differentiation and overall myofiber organization in the BA1-derived jaw muscles was severely disrupted (Fig. 7C'',E'',I'',K''). Furthermore, in some of these CNC-ablated embryos, the BA musculature was missing (Fig. 7G'). Thus, normal cranial skeletal muscle differentiation and myofiber architecture are regulated by the CNC.

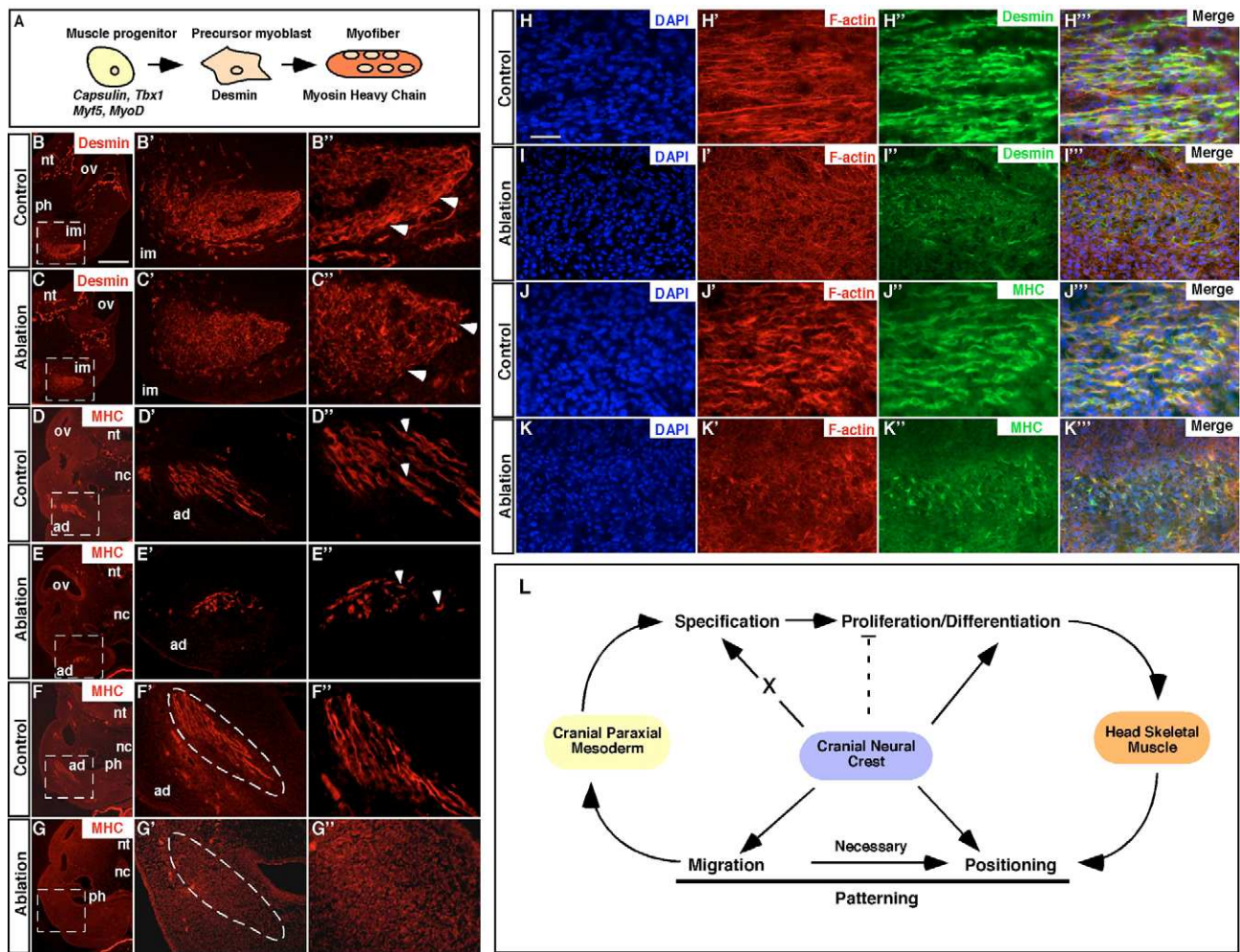


Fig. 7. Myogenic differentiation, myofiber architecture and positioning are perturbed in the absence of the CNC in chick embryos. (A) An illustration of genes that regulate the transition from muscle progenitors to mature myofibers, adapted from Tajbakhsh (Tajbakhsh, 2005). (B-G') Immunofluorescence stainings on transverse sections of E4.5-5 control (B,D,F) or CNC-ablated embryos (C,E,G) for the indicated muscle markers desmin and myosin heavy chain (MHC). The typical organization of BA1-derived jaw muscle fibers is clearly seen in the controls (B',D'; $n=5/5$), but is much less visible in the ablated embryos (C',E'; $n=3/4$). Note the absence of the mandibular adductor in the ablated embryo (compare G' with F'). (H-K'') Higher resolution images of BA1 myofibers: F-actin (phalloidin, red) filaments (H',I',J',K'); desmin, green (H'',I''); MHC, green (J'',K''). DAPI (blue) stains nuclei (H,I,J,K). (L) A model illustrating the multiple roles played by the CNC in the regulation of head skeletal muscles in vertebrate embryos. ad, mandibular adductor; im, intermandibular; nc, notochord; nt, neural tube; ov, otic vesicle. Scale bars: in B, 0.2 mm for C,D,E,F,G, 0.1 mm for B',C',D',E',F',G' and 50 μ m for B'',C'',D'',E'',F'',G'' and 66 μ m for H'-K'''.

Taken together, our findings demonstrate that in vertebrates, although early myogenic specification is CNC-independent, the patterning, migration, proliferation and differentiation of skeletal muscle progenitors are all influenced by CNC cells (Fig. 7L). Furthermore, the early effects of CNC cells on myoblast migration, proliferation and the onset of differentiation could impact upon muscle fiber morphogenesis at later developmental stages. In summary, our results demonstrate that during vertebrate embryogenesis, CNC cells play varying roles in the regulation of skeletal muscle precursors during craniofacial development.

DISCUSSION

Different intrinsic and extrinsic regulatory mechanisms control the developmental programs of trunk and cranial muscle (Hacker and Guthrie, 1998; Kelly et al., 2004; Lu et al., 2002; Mootoosamy and Dietrich, 2002; Noden et al., 1999; Rudnicki et al., 1993; Tajbakhsh

et al., 1997; Tzahor et al., 2003; von Scheven et al., 2006). In the present study, our results have provided insights into the extrinsic regulatory mechanisms that affect head muscle formation, by focusing on the crosstalk between CNC and CPM during myoblast specification, migration, patterning and differentiation.

In vertebrates, CNC cells contribute to the majority of the skeletal and connective tissue within the head but not the muscle fibers, which originate from the mesoderm. However, the tight anatomical proximity between CNC and skeletal muscle precursors, as well as experimental evidence (Couly et al., 1992; Ericsson et al., 2004; Kontges and Lumsden, 1996; Noden, 1983a; Noden, 1983b; Olsson et al., 2001; Schilling and Kimmel, 1997), have led to suggestions that CNC cells play an indirect role during head muscle formation. Explicitly how CNC cells control head muscle patterning, and whether CNC cells promote myogenic differentiation, are issues that remain unresolved. Although our previous study provided evidence

that in the chick, CNC cells promote myogenic differentiation in vitro (Tzahor et al., 2003), a recent report by von Scheven et al. suggested that CNC cells are dispensable for early cranial muscle differentiation (von Scheven et al., 2006).

Our latest findings concerning head skeletal muscle specification, patterning and differentiation in three mouse genetic models: complete loss of a specific population of CNC cells (*Hoxa1/Hoxb1-3* *RARE*), along with defects in CNC cell differentiation and migration (*CA-β-catenin/Wnt1-Cre* and *Twist*), in combination with loss-of-function experiments in the chick, demonstrate that CNC cells regulate skeletal muscle patterning and differentiation in vivo. These results highlight the multiple and dynamic interactions between mesoderm and neural crest cells, crucial to our understanding of head muscle development as well as craniofacial evolution, diversity and pathogenesis.

Myogenic specification

In this study, we show that both capsulin and *Tbx1* were expressed in BA2 of *Hoxa1/Hoxb1-3* *RARE* double mutants, despite the lack of CNC cells. We further demonstrate that early myogenic markers are expressed (although mispatterned) in CNC-ablated chick embryos. These findings, in combination with other studies in amphibians (Ericsson et al., 2004; Olsson et al., 2001) and chick (Tzahor et al., 2003; von Scheven et al., 2006), strongly support the idea that CNC cells are not necessary for the early specification of the skeletal muscle lineage in vertebrates. However, our data clearly demonstrate that CNC cells are involved in diverse aspects of cranial muscle patterning following specification of the myogenic cells.

Head muscle patterning

Our analyses of the skeletal muscle markers in *Twist* mutants demonstrated pronounced defects in the expression of myogenic genes in the head region. These results imply that the location and/or the differentiation of the CNC affect the patterning of the adjacent skeletal muscle markers in a non-cell-autonomous manner. We demonstrated that *Twist* is expressed in CNC cells between E9-E10.5; however, we cannot rule out the possibility that there is a transient expression of *Twist* in the head mesoderm prior to CNC delamination. Tissue-specific knockout of *Twist* in CNC cells should clarify its exact, direct or indirect, impact on myogenesis.

How does *Twist*, a bHLH transcription factor expressed in the CNC cells, affect skeletal muscle formation in the adjacent mesodermal cells? One possibility is that *Twist* might regulate the cell adhesion properties of the CNC cells, and these cells, in turn, could influence skeletal muscle patterning. Indeed, it was recently shown that *Twist* directly regulates the expression of members of the cadherin family of adhesion molecules during tumor development and metastasis (Yang et al., 2004). The observation that cadherin molecules, normally expressed by both mesoderm and CNC cells, were altered in *Twist* mutants may provide a clue as to the nature of the molecular mechanisms underlying the crosstalk between the CNC and skeletal muscle precursors. Interestingly, there is some evidence that myogenesis can be regulated by cell-cell contact mediated by the cell surface receptors CDO and BOC, both of which are related to the cadherin family (Cole et al., 2004).

Using a Cre/loxP system in which a constitutively active form of β-catenin (Harada et al., 1999) was specifically expressed in neural crest cells, it was shown that the Wnt/β-catenin signaling pathway induced sensory neurogenesis by acting instructively on neural crest progenitors while, at the same time, blocking other CNC-derived cell types (Lee et al., 2004). We show that capsulin, *Tbx1* were aberrantly expressed in the BAs of *CA-β-catenin/Wnt1-Cre* mutants,

whereas the myogenic markers, *MyoD* and *Mgn*, were not detected in these mutant embryos. These findings demonstrate that fate specification of CNC progenitors is tightly coupled to the patterning and differentiation of the skeletal muscle progenitors.

CNC ablation experiments in the chick corroborated our genetic studies in the mouse, by showing that in the absence of CNC cells severe muscle patterning defects were seen. In addition, mesoderm cells migrated in an abnormal manner in the CNC-ablated embryos. Based on these results, we propose that in the absence of the CNC, the axial registration of the CPM is disrupted in either an active or a passive manner.

It is well-documented that the axial registration between the CNC and the CPM is maintained as both cell populations remain coherent throughout their migration and subsequent musculoskeletal morphogenesis (Evans and Noden, 2006; Grammatopoulos et al., 2000; Hacker and Guthrie, 1998; Kontges and Lumsden, 1996; Trainor and Tam, 1995). The CNC cells, which anatomically envelop the mesodermal core within the BAs, create barriers to mesodermal cell movement, thus preventing the mixing of mesoderm cells from different axial levels (Noden and Trainor, 2005; Trainor and Tam, 1995). In the absence of these CNC barriers, it is conceivable that abnormal migration of mesoderm cells could occur, resulting in mixing of the normally separate BA streams, and a corresponding disruption of the axial registry. The abnormal migratory behaviors of CPM cells in the chick model could be attributed to the lack of a steric hindrance by the CNC cells, or because of their active (anterior) migration in response to signals from BA1.

Analogous to the head muscles, limb muscle patterning is dependent upon signals from the surrounding skeletogenic mesenchyme derived from the lateral plate mesoderm (Kardon et al., 2003), although in the head, most of the skeletogenic mesenchymal cells are of CNC origin. Ectopic activation of the Wnt/β-catenin pathway in limb mesoderm induced ectopic limb muscles in regions where myotubes do not normally differentiate (Kardon et al., 2003). Conversely, in the head we showed that *Frzb*, a Wnt antagonist, promoted *MyoD* expression in vitro and in vivo (Tzahor et al., 2003). Thus, muscle patterning is extrinsically controlled by the surrounding mesenchymal cells in both the head and the limb, although these signals seem to play distinct roles in each compartment.

Skeletal muscle proliferation and differentiation

We previously demonstrated that CNC induced myogenic differentiation in CPM explants, although in vivo ablation of CNC cells did not significantly affect the expression of *Myf5* (Tzahor et al., 2003; von Scheven et al., 2006). It remains possible that CNC cells could affect myogenic differentiation downstream of *Myf5*. We now show that *Myf5* expression (RNA and protein) is upregulated following CNC ablation in chick embryos. Furthermore, in the *CA-β-catenin/Wnt1-Cre* model, myogenesis seems to be initiated in BA1-2 (low levels of *Myf5* were detected in these areas); however, *MyoD* and *Mgn*, which are downstream genes, failed to be activated in these mutants. These findings are consistent with a previous study demonstrating that *Myf5* expression during limb myogenesis correlates with myoblast proliferation, whereas *MyoD* acts at a later developmental stage, during post-mitotic differentiation (Delfini et al., 2000).

Along these same lines, we further demonstrate that the upregulation of *Myf5* could be linked to increased cell proliferation (observed by *Myf5*⁺/*BrdU*⁺ co-staining) in the BAs of the CNC-ablated embryos. This finding suggests that CNC cells regulate

cranial myogenesis by specifically influencing the rate of cell proliferation/differentiation within the myogenic core. Thus, the progression of myoblasts through differentiation appears to be controlled by the CNC to ensure myogenesis at an appropriate place and time during craniofacial development. In the absence of CNC, some muscle precursor cells presumably fail to exit the cell cycle and to undergo terminal myogenic differentiation.

Interestingly, ablation of the cardiac neural crest cells, a distinct population of neural crest cells originating from the caudal hindbrain (Kirby et al., 1983), resulted in a similar increase in cell proliferation (Waldo et al., 2005), which was attributed to increased Fgf8 signaling in the ventral pharynx (Hutson et al., 2006). We show that *Fgf8* is upregulated in the ectoderm of the BAs in the CNC-ablated embryos. This observation is in line with our in vitro results and in vivo Fgf8 bead application (von Scheven et al., 2006), which demonstrate the reduced myogenic differentiation capacity of this signaling pathway. We propose that CNC ablation induces *Fgf8* upregulation in the BA ectoderm. This, in turn, increases cell proliferation and delays differentiation. Furthermore, in a striking similarity to the cranial mesoderm, mesoderm cells from the secondary heart field failed to migrate into the outflow tract after cardiac neural crest ablation in chick embryos (Waldo et al., 2005). Thus, failure of mesoderm precursors to migrate ventrally at the appropriate time resulted in ectopic sites of cardiac (Waldo et al., 2005) and skeletal muscle differentiation (this study). We suggest that CNC-dependent regulation of mesoderm proliferation and migration (presumably mediated by Fgf8 signaling) constitutes a general regulatory mechanism during vertebrate development.

The increased Myf5⁺/BrdU⁺ co-staining in the head mesoderm compared with the trunk is consistent with the delayed differentiation of the head versus the trunk musculature (Noden et al., 1999). Likewise, it has been suggested that the head mesoderm expresses high levels of putative negative regulators for myogenic differentiation (Bothe and Dietrich, 2006). Indeed, we observed increased expression of capsulin [and *MyoR* (also known as *Msc* – MGI), data not shown] in the CNC-ablated chick and mouse embryos. The pronounced increase in cell proliferation following CNC ablation could explain the significant reduction in late muscle differentiation markers. Immunofluorescence analyses of desmin and MHC in CNC-ablated chick embryos as well as *MyoD* and *Mgn* expression in *CA-β-catenin/Wnt1-Cre* mouse mutants indicate that myogenic differentiation, as well as myofiber architecture and positioning, is regulated by CNC cells.

In summary, our study on craniofacial muscle development in mouse and chick embryonic models has clarified the extent to which the myogenic program is controlled by extrinsic environmental signals. We provide direct evidence that CNC cells play diverse and crucial roles during skeletal muscle formation in vertebrates (Fig. 7L). The appearance of early myogenic markers following surgical ablation of the CNC in chick embryos, or genetic ablation of CNC cells in mouse embryos, shows that early specification of the skeletal muscle lineage is not dependent upon the presence of CNC cells. However, the subsequent migration of skeletal muscle progenitors, along with their patterning, proliferation and differentiation, are tightly controlled by CNC cells. Our findings also demonstrate that other tissues and signals are capable of promoting skeletal muscle differentiation in the head, in the absence of CNC cells. We therefore propose that CNC cells provide guidance cues that enable muscle precursor cells to migrate to the correct positions in the head, and to resume myogenic differentiation in a coordinated manner.

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